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One-Step Multiplex PCR Assay for Differentiating Proposed New Species "*Clostridium neonatale*" from Closely Related Species

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"*Clostridium neonatale*" sp. nov., previously involved in an outbreak of neonatal necrotizing enterocolitis, was recently proposed as a new species of the *Clostridium* genus *sensu stricto*. We developed a one-step multiplex colony PCR for *C. neonatale* identification and investigated *C. neonatale* intestinal colonization frequency in healthy preterm neonates.

In 2002, an outbreak of neonatal necrotizing enterocolitis (NEC) occurred in a Canadian neonatal intensive care unit (1). Blood cultures from three out of six premature neonates grew the same strain proposed to belong to a novel species of *Clostridium*, "*Clostridium neonatale*." However, *C. neonatale* was not formally classified as a new species, resulting in the absence of data about the isolation, identification, or clinical significance of this species. Very recently, based on a polyphasic study combining phylogenetic analysis and phenotypic characterization, we clarified the status of *C. neonatale* by demonstrating that it is a new species belonging to cluster I of the *Clostridium* genus *sensu stricto* (2). Particularly, this study permitted the differentiation of *C. neonatale* from another *Clostridium* species involved in NEC, *Clostridium butyricum*. Indeed, *C. butyricum* has frequently been recovered from biological samples of premature neonates suffering from NEC (3–7). Additionally, in quail and chicken animal models of NEC, *C. butyricum* was shown to be responsible for NEC-like lesions (8–11). *C. neonatale* and *C. butyricum* were reported to be significantly overrepresented among colonic mucosal samples from premature piglets with NEC (12).

The purpose of the current study was to develop a one-step multiplex colony PCR for *C. neonatale* identification as an alternative tool to 16S rRNA gene sequencing and matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS). Next, this PCR was used to screen strains isolated from the fecal samples of preterm neonates, leading to a report of *C. neonatale* intestinal colonization frequency for the first time.

Strains included in this study were isolated from fecal samples, as previously described (13), and belong to our laboratory collection (EA4065 Ecosystème Intestinal, Antibiotiques, Probiotiques, Université Paris Descartes, Paris, France). For this study, 190 clostridial strains were isolated from fecal samples of 88 healthy preterm neonates (collected at an average age of 8.6 weeks of life [range, 1 to 85 weeks]). The strains were grown on Columbia agar medium (Oxoid, Dardilly, France) supplemented with 5% sheep blood (vol/vol) for 24 h at 37°C in an anaerobic chamber (80% N_2, 10% CO_2, and 10% H_2) (AES Chemunex, Bruz, France). Strains were initially identified using classical microbiological approaches, including rapid ID 32A strips (bioMérieux, Marcy l'Etoile, France). Out of the 190 clostridial isolates, we focused on 100 strains that resulted in either good identification scores for rapid ID 32A strip *C. butyricum*/*C. beijerinckii* (>87%) (apiweb identification software version 3.2) or other clostridial species with low-percentage identification scores (<80%). Unambiguous identification of the 100 strains was performed using partial 16S rRNA gene sequencing and MALDI-TOF MS analysis of whole-colony spectral fingerprints, as previously reported (2). Based on this accurate identification, we developed a one-step multiplex PCR scheme for the identification of *C. neonatale*, *C. butyricum*, and *C. beijerinckii*. Indeed, if *C. beijerinckii* has not been associated with NEC pathogenesis or isolated from the human gut, it was included in the present study as a species that is closely related to *C. butyricum* (14).

C. neonatale, *C. butyricum*, and *C. beijerinckii* species-specific PCR primers (Table 1) were designed based on a previously re-

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TABLE 1 Primers used in this study

Primer	Sequence (5′–3′)	Target	Product size (bp)	Gene (accession no.)
P2051F	CATTGCATGGAAAATTTGGA	"*C. neonatale*"	310	*ddl* (KF683394)
P2052R	GCTTCTGCTACGCATTCCTC			
P2053F	TCATCAATACAATGGGCTAGAGAA	*C. butyricum*	253	*ctps* (KF683390)
P2054R	CTTCGTATGAAGTGCTTTCCA			
P2055F	AAGGAAAAGTATGAAGTGCTACCAA	*C. beijerinckii*	232	*ddl* (KM452709)
P2056R	TCCCATTTGCCTCTAAAACG			

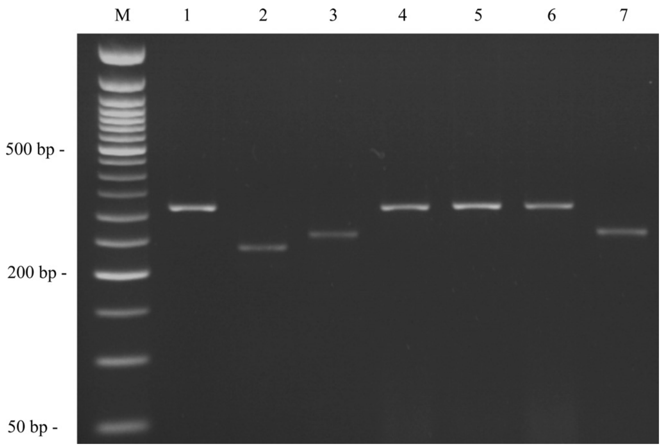

FIG 1 Gel electrophoresis of PCR products (5 μl) from the one-step colony multiplex PCR. Lane M, molecular marker (50 bp; New England BioLabs, Évry, France); lane 1, *C. neonatale* ATCC BAA-265, amplicon of 310 bp; lane 2, *C. butyricum* VPI 3266^{T}, amplicon of 253 bp; lane 3, *C. beijerinckii* VPI 5481^{T}, amplicon of 232 bp; lanes 4 to 6, example of *C. neonatale* clinical isolates, amplicon of 310 bp; lane 7, example of *C. butyricum* clinical isolates, amplicon of 253 bp.

TABLE 2 Reference strains used in this study

Species	Source
Clostridium cluster I *sensu stricto*	
C. aurantybutyricum	ATCC 17777 ^{T}
C. butyricum	VPI 3266^{T}
C. carnis	ATCC 25777^{T}
C. cochlearium	ATCC 17787 ^{T}
C. paraputrificum	ATCC 25780 ^{T}
"*C. neonatale*"	ATCC BAA265
C. oceanicum	DSM 1290 ^{T}
C. perfringens	ATCC 13124 ^{T}
C. sporogenes	ATCC 3584 ^{T}
C. sordellii	ATCC 9714 ^{T}
C. tetanomorphum	DSM 4474 ^{T}
C. innocuum	ATCC 14501 ^{T}
C. celatum	ATCC 27791 ^{T}
C. tyrobutyricum	ATCC 25755 ^{T}
Other *Clostridium* spp.	
C. bolteae	DSM 15670 ^{T}
Hungatella effluvii (formerly *C. hathewayi*)	DSM 13479 ^{T}
C. difficile	ATCC 9689 ^{T}

ported multilocus sequence typing scheme (2). Bacterial DNA from *C. neonatale* ATCC BAA-265, *C. butyricum* VPI 3266^{T}, and *C. beijerinckii* VPI 5481^{T} reference strains were purified using the InstaGene matrix kit (Bio-Rad, Marnes-la-Coquette, France) and used as the PCR DNA template. PCR amplification was carried out in a total volume of 50 μl containing 1.25 U of *Taq* polymerase (Life Technologies, Saint-Aubin, France), 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton, 2.5 mM $MgCl_2$, 0.5 mM each primer, 0.2 mM deoxynucleoside triphosphates (dNTPs), and 10 ng of purified DNA. The PCR program was conducted as follows: one cycle of 95°C for 10 min, 35 cycles consisting of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, and a final cycle of 72°C for 10 min. PCR products were analyzed by electrophoresis on a 2% agarose gel, followed by ethidium bromide staining. As expected, a unique DNA amplicon was obtained for the *C. neonatale* (310 bp), *C. butyricum* (253 bp), and *C. beijerinckii* (232 bp) reference strains (Fig. 1). No PCR product was obtained with purified DNA from the type strains tested (Table 2) (data not shown).

Next, the multiplex PCR assay was performed on single colonies picked from fresh plates and directly resuspended in the tube containing the PCR mix (final volume, 25 μl). This allowed the correct identification of the three reference strains and all 100 clostridial strains tested, by producing a unique PCR product of the expected size (Fig. 1). The results of the multiplex PCR assay showed 100% agreement with both 16S rRNA gene sequencing and MALDI-TOF MS identification data.

The specificity of the single-colony multiplex PCR assay was verified using the reference strains presented in Table 2. It resulted in the absence of PCR product amplification, except for the *C. neonatale*, *C. butyricum*, and *C. beijerinckii* reference strains. All samples that gave a negative multiplex PCR amplification product were checked by running the 16S rRNA gene PCR product, as previously described (13). As expected, a band of 1,200 bp was obtained. This indicated that the lack of an amplification product reflected the specificity of our protocol rather than the lack of suitable template DNA (data not shown).

When considering the 190 clinical clostridial isolates, *C. neonatale* was identified 27 times, corresponding to a preterm neonate colonization frequency of 14.2% compared to 38.4% for *C. butyricum* ($n = 73$). No *C. beijerinckii* strain was isolated from our samples: this is in agreement with the fact that *C. beijerinckii* has rarely been isolated from human fecal samples.

The heterogeneity of the *Clostridium* genus does not facilitate correct strain identification, which remains laborious and time-consuming. We propose a one-step colony multiplex PCR for the identification of the proposed new *Clostridium* species *C. neonatale* as an easy and fast alternative approach to 16S rRNA gene sequencing and MALDI-TOF MS analysis, which is not always available in all laboratories. This multiplex PCR assay represents a reliable tool to investigate the role and clinical significance of *C. neonatale*, a species that may have been misidentified and underrepresented in previous neonatal necrotizing enterocolitis microbial analyses. Work is in progress to determine the experimental conditions needed to detect *C neonatale* directly from fecal samples by means of real-time PCR. In this study, we also report for the first time *C. neonatale* intestinal colonization frequency in healthy preterm neonates and suggest that this species can be found as a commensal bacterium of the gut microbiota.

REFERENCES

1. **Alfa MJ, Robson D, Davi M, Bernard K, Van Caeseele P, Harding GK.** 2002. An outbreak of necrotizing enterocolitis associated with a novel *Clostridium* species in a neonatal intensive care unit. Clin Infect Dis **35:** S101–S105. http://dx.doi.org/10.1086/341929.
2. **Bouvet P, Ferraris L, Dauphin B, Popoff MR, Butel MJ, Aires J.** 2014. 16S rRNA gene sequencing, multilocus sequence analysis, and mass spectrometry identification of the proposed new species "*Clostridium neonatale.*" J Clin Microbiol **52:**4129–4136. http://dx.doi.org/10.1128/JCM.00477-14.
3. **Howard FM, Bradley JM, Flynn DM, Noone P, Szawatkowski M.** 1977. Outbreak of necrotising enterocolitis caused by *Clostridium butyricum*. Lancet **ii:**1099–1102.

4. **Mitchell RG, Etches PC, Day DG.** 1981. Non-toxigenic clostridia in babies. J Clin Pathol **34:**217–220. http://dx.doi.org/10.1136/jcp.34.2.217.
5. **Kliegman RM, Fanaroff AA.** 1984. Necrotizing enterocolitis. N Engl J Med **310:**1093–1103. http://dx.doi.org/10.1056/NEJM198404263101707.
6. **Sturm R, Staneck JL, Stauffer LR, Neblett WW, III.** 1980. Neonatal necrotizing enterocolitis associated with penicillin-resistant, toxigenic *Clostridium butyricum*. Pediatrics **66:**928–931.
7. **Smith B, Bode S, Petersen BL, Jensen TK, Pipper C, Kloppenborg J, Boyé M, Krogfelt KA, Mølbak L.** 2011. Community analysis of bacteria colonizing intestinal tissue of neonates with necrotizing enterocolitis. BMC Microbiol **11:**73. http://dx.doi.org/10.1186/1471-2180-11-73.
8. **Popoff MR, Szylit O, Ravisse P, Dabard J, Ohayon H.** 1985. Experimental cecitis in gnotoxenic chickens monoassociated with *Clostridium butyricum* strains isolated from patients with neonatal necrotizing enterocolitis. Infect Immun **47:**697–703.
9. **Bousseboua H, Le Coz Y, Dabard J, Szylit O, Raibaud P, Popoff MR, Ravisse P.** 1989. Experimental cecitis in gnotobiotic quails monoassociated with *Clostridium butyricum* strains isolated from patients with neonatal necrotizing enterocolitis and from healthy newborns. Infect Immun **57:**932–936.
10. **Waligora-Dupriet AJ, Dugay A, Auzeil N, Huerre M, Butel MJ.** 2005. Evidence for clostridial implication in experimental necrotising enterocolitis through bacterial fermentation in a gnotobiotic quail model. Pediatr Res **58:**629–635.
11. **Szylit O, Butel MJ, Rimbault A.** 1997. An experimental model of necrotising enterocolitis. Lancet **350:**33–34. http://dx.doi.org/10.1016/S0140-6736(05)66243-5.
12. **Azcarate-Peril MA, Foster DM, Cadenas MB, Stone MR, Jacobi SK, Stauffer SH, Pease A, Gookin JL.** 2011. Acute necrotizing enterocolitis of preterm piglets is characterized by dysbiosis of ileal mucosa-associated bacteria. Gut Microbes **2:**234–243. http://dx.doi.org/10.4161/gmic.2.4.16332.
13. **Ferraris L, Butel MJ, Campeotto F, Vodovar M, Rozé JC, Aires J.** 2012. Clostridia in premature neonates' gut: incidence, antibiotic susceptibility, and perinatal determinants influencing colonization. PLoS One **7:**e30594. http://dx.doi.org/10.1371/journal.pone.0030594.
14. **Magot M, Carlier JP, Popoff MR.** 1983. Identification of *Clostridium butyricum* and *Clostridium beijerinckii* by gas-liquid chromatography and sugar fermentation: correlation with DNA homologies and electrophoretic patterns. J Gen Microbiol **129:**2837–2845.